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VEGF-B IN ISLETS OF LANGERHANS: ROLE IN VASCULAR SIGNALING AND LIPID HANDLING

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Cover image: An islet of Langerhans stained with insulin (green), glucagon (red) and vessel marker CD31 (white).
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VEGF-B IN ISLETS OF LANGERHANS: ROLE IN VASCULAR SIGNALING AND LIPID HANDLING

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谨以此书献给敬爱的奶奶
并谨此纪念我敬爱的爷爷

To my dear grandmother and
In loving memory of my grandfather

ABSTRACT

The discovery of Vascular endothelial growth factor (VEGF)-B's role in endothelial fatty acid uptake and tissue lipid accumulation opened a new arena for exploration of VEGF-B in lipid metabolism. Indeed, later studies demonstrated the beneficial/therapeutic effects of reducing VEGF-B signaling systemically. However, the tissue specific role of VEGF-B signaling has yet to be determined and furthermore the contribution of tissue specific VEGF-B activity to the systemic effects is also unknown to date.

In **paper I**, we investigated the role of local VEGF-B signaling in pancreatic islets by employing a novel mouse model where *Vegfb* in pancreatic β-cells were selectively deleted using Cre/loxP technology. We showed that *Vegfb* is abundantly expressed in both endocrine and exocrine pancreas, but that ablation of *Vegfb* from β-cells does not affect systemic glucose homeostasis or islet lipid uptake under chow or high-fat diet (HFD) conditions. However, deletion of *Vegfb* in pancreatic β-cells increased insulin (*Ins2*) gene expression, which indicates the potential interaction between VEGF-B signaling and transcriptional regulation of insulin, a phenotype warranting further studies.

In **paper II**, we continued to explore the role of pancreatic β-cell derived VEGF-B in systemic homeostasis. In contrast to the mouse model used in paper I, we utilized a mouse model where the human VEGF-B_{167} isoform is overexpressed in pancreatic β-cells (under the rat insulin promoter). In this study, we could demonstrate that overexpression of VEGF-B in pancreatic β-cells does not affect systemic glucose homeostasis under chow or HFD conditions. Unexpectedly, overexpression of VEGF-B in pancreatic islets led to increased plasma triglycerides. This could indicate VEGF-B's involvement in plasma lipoprotein synthesis or degradation, and further studies are needed to elucidate this effect.

The role of VEGF-B in angiogenesis is somewhat ambiguous. To address this question, in **paper III**, we deployed a newly developed intravital imaging protocol where isolated mouse islets were transplanted into the anterior chamber of the eye to facilitate the observation of islet vessel development, pharmacological treatment of tumor islets and effects of VEGF-B overexpression in β-cells. We showed that the advantage of this platform comprises high spatial resolution, real-time monitoring of islet development and feasibility for evaluating efficacy of pharmacological treatments of tumor islets. In addition, we observed that

overexpression of VEGF-B in tumor islets increased tumor angiogenesis but impaired tumor growth, providing additional information about the role of VEGF-B in pathological angiogenesis.

Canonical Wnt/β-catenin signaling is essential for the retinal and cerebral angiogenesis during development. However, little is known regarding the contribution of endothelial β-catenin signaling in postnatal central nervous system (CNS) and potential interaction with other signaling pathways regulating angiogenesis. In **paper IV**, we studied the role of endothelial β-catenin signaling in central nervous system angiogenesis. We demonstrated that impaired Wnt/β-catenin signaling resulted in reduced postnatal retinal- and cerebral angiogenesis, presumably by diminished VEGFR2 expression. We furthermore identified crosstalk between the Wnt/β-catenin and Notch/VEGFA signaling pathways.

In summary, this thesis provides further knowledge about the specific role of VEGF-B signaling in islets of Langerhans, and its effects on systemic glucose homeostasis and lipid handling. Additionally, development of a novel intravital imaging protocol, as well as uncovering of additional roles of endothelial Wnt/β-catenin signaling in CNS, constitute parts of this thesis.

LIST OF SCIENTIFIC PAPERS

I. **Frank Chenfei Ning**, Nina Jensen, Jiarui Mi, William Lindström, Mirela Balan, Lars Muhl, Ulf Eriksson, Ingrid Nilsson & Daniel Nyqvist.
VEGF-B ablation in pancreatic β-cells upregulates insulin expression without affecting glucose homeostasis or islet lipid uptake.
Scientific Reports 10, 923 (2020) doi.org/10.1038/s41598-020-57599-2

II. **Frank Chenfei Ning**, Mirela Balan, Jiarui Mi, Ulf Eriksson, Ingrid Nilsson & Daniel Nyqvist.
Overexpression of VEGF-B in pancreatic β-cells does not affect glucose homeostasis but increases plasma triglycerides.
Manuscript

III. Mirela Balan, Marta Trusohamn, **Frank Chenfei Ning**, Stefan Jocob, Kristian Pietras, Ulf Eriksson, Per-Olof Berggren & Daniel Nyqvist.
Noninvasive intravital high-resolution imaging of pancreatic neuroendocrine tumours.
Scientific Reports 9, 14636 (2019) doi:10.1038/s41598-019-51093-0

IV. Agnieszka Martowicz, Marta Trusohamn, Nina Jensen, Joanna Wisniewska-Kruk, Monica Corada, **Frank Chenfei Ning**, Julianna Kele, Elisabetta Dejana & Daniel Nyqvist.
Endothelial β-Catenin Signaling Supports Postnatal Brain and Retinal Angiogenesis by Promoting Sprouting, Tip Cell Formation, and VEGFR (Vascular Endothelial Growth Factor Receptor) 2 Expression.
Arteriosclerosis, Thrombosis, and Vascular Biology, 39, 2273-2288 (2019). doi:10.1161/ATVBAHA.119.312749

医，以人为本，以德为尚，以术为精，仁爱救人

CONTENTS

LIST OF ABBREVIATIONS

ACE	Anterior chamber of the eye
CK1	Casein kinase
DAG	Diacylglycerol
DKD	Diabetic kidney disease
Dvl	Dishevelled
ER	Endoplasmic reticulum
ERRα	Estrogen-related receptor α
FA	Fatty acid
FABP	Fatty acid binding protein
FAT	Fatty acid translocase
FATP	Fatty acid transport protein
FFA	Free fatty acid
FFAR	Free fatty acid receptor
FZD	Frizzled
GPR	G-protein-coupled receptor
GSIS	Glucose stimulated insulin secretion
GSK3	Glycogen synthase kinase 3
LCFA	Long chain fatty acid
LDL	Low density lipoprotein
LRP	Low density lipoprotein receptor-related protein
MAG	Monoacylglycerol
NADH	Nicotinamide adenine dinucleotide, reduced form
NRF1	Nuclear respiratory factor 1
NRP 1/2	Neuropilin 1/2
PGC-1α	Peroxisome proliferator activated receptor γ coactivator 1α
PPARγ	Peroxisome proliferator activated receptor γ
ROS	Reactive oxygen species
SCFA	Short-chain fatty acid
TCF	T cell factor
TG	Triglyceride
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VPF	Vascular permeability factor

INTRODUCTION

I

VASCULAR ENDOTHELIAL GROWTH FACTOR B

1.1 Biology, Tissue Distribution and Regulation of VEGF-B

1.1.1 The Biology of VEGF-B

The vascular endothelial growth factor (VEGF), previously known as the vascular permeability factor (VPF), was first discovered by Senger et al. in 1983 through the study of tumor ascites fluids secreted by a number of tumor cell lines[4]. Later that year, Napoleone Ferrara successfully isolated VEGF and indicated its role in angiogenesis[5]. The VEGF family consists of six mammalian glycoprotein growth factors: VEGF-A (or VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor (PIGF)[6]. They mainly bind to three different tyrosine kinase receptors that are expressed by vascular and lymphatic endothelial cells with an overlapping pattern: VEGFR1, VEGFR2 and VEGFR3[7]. VEGF is essential for vasculogenesis during embryonic development and also for angiogenesis under both physiological and pathological conditions[6,7]. In endothelial cells, the common semaphorin receptors Neuropilin-1 (NRP1) and NRP2 can also act as co-receptors for several of the VEGFs[8] (Figure 1). The VEGF ligands and their receptors mediate signaling in both autocrine and paracrine fashion[9-11].

VEGF-B, a member of the VEGF family encoded by the *Vegfb* gene, was first discovered in 1996 and is highly conserved in mammalian species[12,13]. Structurally, the *Vegfb* gene contains seven exons and due to alternative splicing on splice acceptor sites in exon 6, the *Vegfb* gene

gives rise to two protein isoforms, VEGF-B_{167} and VEGF-B_{186}[12,14]. Both VEGF-B isoforms are expressed in various tissues and binds specifically to VEGFR1 and NRP1 on endothelial cells[15,16]. VEGF-B_{167} is the prevalent isoform and contains a highly basic heparin-binding carboxyl terminus, which promotes binding to cell surface heparan sulfate proteoglycans in the extracellular matrix[17]. In contrast, VEGF-B_{186} contains a hydrophobic carboxyl terminus and is freely diffusible. Unlike VEGF-B_{167}, proteolytic cleavage of the VEGF-B_{186} carboxyl terminus is required before binding to the co-receptor NRP1[15].

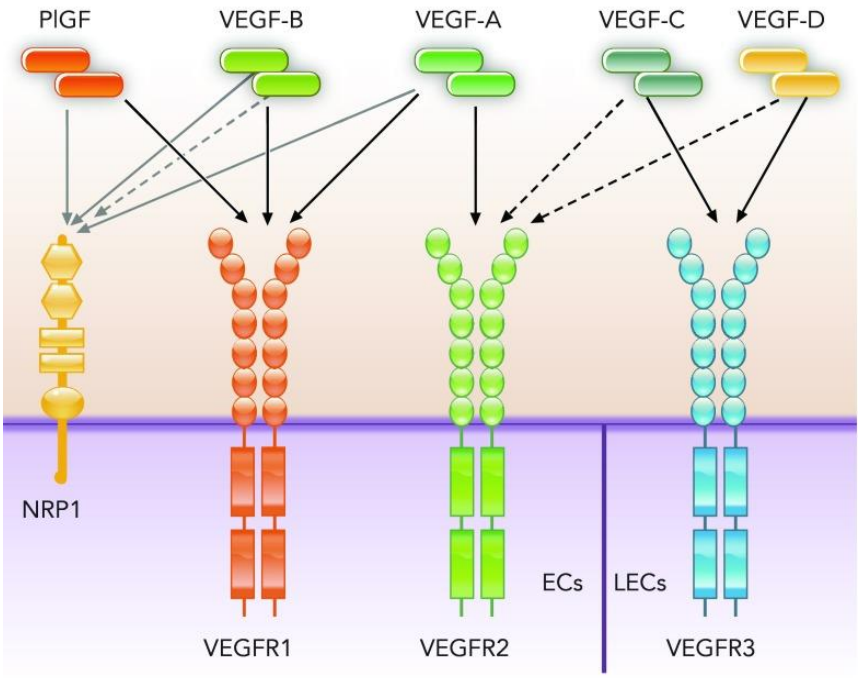

Figure 1. Schematic diagram of VEGF ligands and receptors

Partially overlapping binding pattern of VEGF ligands to their receptors. VEGF-A, VEGF-B and placental growth factor (PIGF) also bind the co-receptor NRP1. *(Modified image from Hagberg et. al, Physiology, 2013[1])*

1.1.2 Tissue Distribution

Under physiological conditions, VEGF-B is widely expressed in adult mouse and human tissue with abundant expression in tissues with high metabolic capacity, such as the myocardium, skeletal muscle, vascular smooth muscle, brown adipose tissue, kidney and brain, among which heart and skeletal muscle have the highest expression[9,13,18-20]. However, endothelial cells seem to lack VEGF-B expression[20]. VEGF-B_{167}, which accounts for over 80% of total VEGF-B transcripts is expressed in most tissues in the mouse[17]. VEGF-B_{186} isoform on the other hand, exhibits a much lower expression level and is expressed in a limited

number of organs[17]. Interestingly, contrary to the generally low expression of the VEGF-B_{186} isoform in the normal healthy state, upregulated expression of VEGF-B_{186} was found in both human and mouse tumor cell lines[17].

With regard to VEGF-B specific expression in pancreas, VEGF-B has been shown to be expressed in pancreatic islets, including the β-cells, similar to other members of the VEGF family[21,22]. Single cell transcriptome studies on mouse and human islets also revealed that *Vegfb* is expressed in all types of endocrine cells and also in acinar cells[23,24]. In our recent study (listed as paper 1 in this thesis), we have additionally demonstrated that VEGF-B is expressed both in endocrine and exocrine tissues with similar abundance using RNA *In Situ* hybridization and that the expression level in pancreatic islets is approximately 10-fold lower compared to heart and skeletal muscle[25].

Under pathological conditions, VEGF-B has been suggested to play a role in neoangiogenesis due to higher expression level in certain cancer types[26]. However, VEGF-B expression level remains constant through hyperplastic progression or angiogenic phase when crossed with pancreatic cancer inducing mouse model (RIP1-Tag2)[21]. VEGF-B transcripts were also found in various human cancers such as breast[27] and ovarian cancer[28], adenocarcinoma[29], and sarcoma, lymphoma and melanoma[26]. Furthermore, a decreased VEGF-B expression were reported in dilated cardiomyopathy and ischemic heart disease[30].

1.1.3 Regulation of VEGF-B

Current knowledge regarding transcriptional regulation of VEGF-B expression is still limited. Of note, bioinformatic analysis revealed that *Vegfb* is tightly co-expressed with a cluster of nuclear-encoded mitochondrial genes, in particular NADH dehydrogenase 1a subcomplex 5 (*Ndufa5*) and cytochrome c (*Cycs*)[19]. Notably, these two genes are regulated by peroxisome proliferator activated receptor γ co-activator 1α (PGC-1α), a master regulator of mitochondrial energy metabolism[31]. Therefore, these data hinted to the possibility that PGC-1α might also regulate VEGF-B expression. A later study by Mehlem et al. indeed demonstrated that *Vegfb* expression is regulated by PGC-1α[32]. In response to external stimuli such as exercise, fasting and cold, PGC-1α is induced and activates several transcription factors, such as nuclear respiratory factor 1 (NRF1), PPARγ and estrogen-related receptor α (ERRα), to exert its effects[32]. PGC-1α activation during fasting/exercise induces mitochondrial biogenesis, facilitates cellular fatty acid (FA) uptake for β-oxidation, and

promotes angiogenesis by increasing the expression of *Vegfa* and *Vegfb*[32]. Having VEGF-B expression under the control of PGC-1α fits very well from an evolutionary point of view in order to ensure sufficient nutrient supply to tissues with high metabolic activities in order to maintain tissue energy homeostasis under challenging conditions.

Unlike other members of the VEGF family, VEGF-B expression was originally not thought to be induced by hypoxia due to lack of a hypoxia-response element sequence in the *Vegfb* promoter region[33]. However, a recent study demonstrated hypoxia-induced VEGF-B expression in mouse retina and that VEGF-B signaling via VEGFR1 plays a role in pathological retinal neovascularization[34]. Therefore, further studies are warranted to further validate the role of hypoxia in the regulation of VEGF-B expression.

1.2 Role in Angiogenesis, Fatty Acid Uptake and Metabolism

1.2.1 Role of VEGF-B in Angiogenesis

Attributable to the high sequence homology and overlapping receptor binding patterns to VEGF-A[35,36], VEGF-B was originally thought to be an angiogenic factor, and VEGF-B was initially shown to stimulate endothelial cell growth *in vitro*[13]. However, unlike mouse models exhibiting VEGF-A or VEGF-C deficiency that are embryonically lethal with vascular defects, VEGF-B deficient mice are healthy and fertile[37-40]. Pathological angiogenesis in most organs studied are also not affected by VEGF-B deficiency[41]. Additional studies in deficient mice also have demonstrated that VEGF-B is neither required for vessel formation in proliferative retinopathy[39] nor for the blood vessel remodeling in pulmonary hypertension[40].

Strategies to study VEGF-B´s angiogenic potential by transgenic overexpression *in vivo* have mostly turned out negative[42,43], in contrast to most other VEGF family members that exhibit either angiogenic or lymphangiogenic properties after transgenic overexpression, such as VEGF-A[44-46], VEGF-C[47], VEGF-D[48] and VEGF-E[49]. For instance, ectopic overexpression of VEGF-B under the Tie2 promoter in endothelial cells did not induce angiogenesis *in vivo*, and instead it was suggested that endothelial cell specific overexpression of VEGF-B potentiated postnatal vessel growth rather than initiation of vessel growth[43]. The use of adenoviral vectors for delivering VEGF-B into muscle and periadventitial tissue did not trigger vessel growth either[50,51]. In addition, although VEGF-B overexpression in the skin increased capillary diameter, the effect on blood vessel density was modest[42].

In the eye, overexpression of VEGF-B by gene transfer has on the contrary been shown to promote pathological retinal and choroidal neovascularization in mice[52]. In addition, Robciuc et al. demonstrated a novel function of VEGF-B in adipose tissue, where gene transfer of the VEGF-B_{186} isoform via an adeno-associated viral vector resulted in increased vascularity and perfusion of the adipose tissue, correlating with an improved insulin sensitivity and overall metabolic condition[53]. The positive effect of VEGF-B overexpression on angiogenesis was attributed to increased ligand occupation of VEGFR1, resulting in increased bioavailability of VEGF-A to VEGFR2 and activation of proangiogenic VEGF-A/VEGFR2 signaling[53].

1.2.2 Fatty Acid Uptake and Metabolism

Hagberg et al. demonstrated that stimulation of endothelial cells with both VEGF-B isoforms induce expression of fatty acid transporter protein (FATP)-3 and 4 both at mRNA and protein level[19]. Stimulation of cultured endothelial cells with VEGF-B also increased the cellular uptake of long chain fatty acids (LCFAs), and that this process could be blocked by silencing the expression of FATP3 and FATP4 using siRNA[19,54]. Further *in vitro* studies revealed that treatment of cultured endothelial cells with VEGFR1 and NRP1 neutralizing antibodies blocked expression of FATP3, suggesting that both receptors were required for VEGF-B mediated FATP expression[19]. *In vivo* studies have also shown that overexpression of VEGF-B by adenoviral delivery is capable of inducing FATP expression[19]. Depletion of *Vegfb* in mice resulted in decreased expression of FATP3 and FATP4 in endothelial cells and decreased lipid accumulation in the heart, skeletal muscle and brown adipose tissue. Instead lipids were redirected to white adipose tissue[19]. In addition, both VEGFR1 and endothelial cell-specific NRP1 deficient mice exhibit defective FA uptake to peripheral tissues, indicating that both receptors are essential for VEGF-B mediated FATP expression *in vivo*[19,20]. Taken together, these data indicated that VEGF-B controls endothelial FA uptake through a paracrine regulation of vascular FATPs via VEGFR1 and NRP1 signaling (Figure 2).

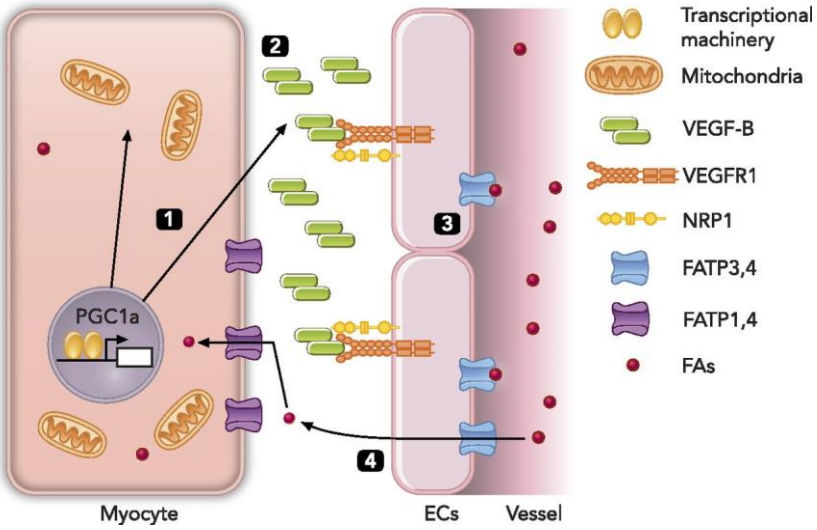

Figure 2. Schematic illustration of VEGF-B in trans-endothelial FA transport

1: VEGF-B and mitochondrial proteins under the control of PGC1 are co-expressed in tissue cells to co-ordinate endothelial fatty acid transcytosis and tissue β-oxidation. 2: VEGF-B signals via VEGFR1 and NRP1 on the endothelial cells in a paracrine fashion. 3: Stimulation of endothelial cells with VEGF-B upregulates vascular FATPs expression and induces transport of fatty acids cross the endothelial layer into tissue cells. *(Modified Image from Hagberg et. al, Physiology, 2013[1])*

A follow-up study revealed that VEGF-B deficient mice kept on high fat diet or crossed with type-2-diabetic *db/db* mice display increased insulin sensitivity and improved glucose homeostasis and plasma lipid profile. Furthermore, genetic deletion of *Vegfb* in diabetic mice showed increased cardiac glucose uptake, decreased ectopic lipid deposition in heart and skeletal muscle, and normalized blood glucose. This effect could also be replicated in *db/db* mice by pharmacological inhibition of VEGF-B signaling using neutralizing antibodies[55]. The *db/db* mice showed improved glucose tolerance, β-cell function and preserved pancreatic islet architecture. The overall lipid storage was reduced in heart, skeletal muscle and pancreas, however, not in liver[55], suggesting that targeting VEGF-B signaling can limit peripheral lipid accumulation, hence restore tissue insulin sensitivity and β-cell function. Therefore, VEGF-B is essential for tissue lipid homeostasis and constitute a target for developing a novel treatment against metabolic syndrome. In line with these observations, other studies have also shown similar results. Kivelä et al. observed increased FATP4 expression in rat heart with VEGF-B overexpression and conversely decreased FATP4 expression in rat heart with VEGF-B deficiency[56]. In addition, a study by Karpanen et al. demonstrated that cardiac

specific overexpression of VEGF-B resulted in increased ceramide accumulation in the heart[42].

In a subsequent study, Falkevall et al. further demonstrated that inhibiting VEGF-B mediated FA uptake could be therapeutically beneficial. Using multiple mouse models of diabetic kidney disease (DKD), the study showed that renal VEGF-B expression correlated with DKD disease severity and inhibition of VEGF-B either through genetic deletion or pharmacological inhibition could reduce renal lipotoxicity, prevent renal dysfunction and inhibit DKD development[57].

In the study by Robciuc et al. they also observed that activation of VEGF-B/VEGFR1 pathway by gene transfer could induce weight loss and counteract metabolic complications in obese and insulin resistant mice, suggesting the therapeutic potential for targeting VEGF-B/VEGFR1 pathway for treating metabolic disease[53]. Gene expression data from mice and human revealed high expression of VEGF-B and its receptors in both white and brown adipose tissue[58], suggesting that VEGF-B might play an important role in adipose tissue. In white adipose tissue of VEGF-A deficient mice, VEGF-B, FATPs 1-4 and brown adipose tissue specific genes were upregulated and the mice also have lower body weight when fed on high fat diet[59]. Another study has shown that cold stimulation downregulates VEGF-B and FATP3 expression in brown adipose tissue[60]. However, both studies were observatory, and the precise mechanism regarding this regulation is still unclear.

II

ISLETS OF LANGERHANS AND LIPIDS

2.1 Islet Anatomy, Biology and Vasculature

2.1.1 The Anatomy and Biology of Islets of Langerhans

The pancreatic islets, also referred to as the islets of Langerhans, were first discovered by the German anatomist Paul Langerhans in 1869[61]. Dispersed inside the pancreas, the islets represent the endocrine part of the pancreas and consist of at least five different hormone producing endocrine cell types, including the glucagon producing α-cells, insulin producing β-cells, pancreatic polypeptide producing γ-cell and somatostatin producing δ-cells. β-cells account for the majority of the islet cells (70%) and the hormones produced by the different endocrine cells are directly secreted into the circulation to exert their respective functions.

The distribution of the different endocrine cells within the islet are species dependent[62]. In human islets the β-cells are intermingled with other endocrine cells, whereas β-cells in rodent islets are surrounded by a ring of α-cells[63]. The islets are defined as micro-organs with a highly specialized and dense capillary vascular network. Islets are spread out over the entire pancreas and constitute around 4.5% of the total pancreas volume[64]. Despite a rather small volume, the islets receive around 10-15% of the pancreatic blood flow. The dense islet vascular network has an important physiological function as it facilitates the rapid exchange of glucose and

insulin between *e.g.* β-cells and blood, thereby supporting glucose sensing and insulin secretion[62].

One of the important functions of pancreatic islet is to rapid secreting insulin/glucagon into circulation in response to physiological signals. β-cells as the majority of the cell population in islet, their ability to secreting insulin is essential in maintaining systemic glucose homeostasis. In order to achieve this rapid response, insulin is released from stored granules via exocytosis through two phases[65]. The first phase characterized by rapid release of insulin secretory granule from readily insulin granule pool in response to elevated blood glucose, whereas second phase is characterized by a sustained slow release of newly formed granule[66]. Dysfunction in insulin release and failure to maintain glucose homeostasis have been implicated in the onset of both type 1 and 2 diabetes mellitus.

2.1.2 The Vasculature of the Islets of Langerhans

The establishment of the specialized endothelium within the islet vasculature is a direct result of micro-environmental signals, especially proteins from the VEGF family. VEGF-A has been shown to play a crucial role in the formation of the islet vasculature[67]. During embryonal development, differentiating endocrine cells start to secrete ample amounts of VEGF-A, which in turn attracts endothelial cells to the site of the developing islets. The endothelial cells in turn secrete angiocrine factors, causing neighboring β-cells to proliferate, and this reciprocal interaction shapes the outline of the islet vascular bed resulting in a dense capillary network[68,69]. VEGF-A is not only essential for formation of the islet vasculature, but also for the development of endothelial pores, denoted fenestrae[65]. Compared to exocrine tissue, islet capillaries are highly permeable, which enables the rapid exchange of macromolecules and prompt insulin delivery into the vascular space, as well as for efficient interstitial flow in mature islets[68,70]. To further understand the role of VEGF-A in islet vascularization and fenestration, a number of studies using transgenic mouse models where VEGF-A was either specifically deleted in β-cells or in the entire pancreatic epithelium has been evaluated[22,68,70]. VEGF-A deficient islets exhibit a significantly reduced vascular network, capillary size and endothelial cell permeability; 10 times less fenestrated than control islets, these mice are also glucose intolerant[67,70], presumably due to a severely reduced insulin output into the vascular system[68]. However, VEGF-A deficiency did not completely suppress blood vessel formation, indicating that other growth factors are also involved in islet vascularization[70].

One of the important functions of the capillary network in islets is to support efficient secretion of different metabolic hormones directly into the blood stream[71-73]. Understanding the explicit route that the blood will follow within the islet is therefore of great importance in order to understand the communication between different islet cells. Currently, the precise organization of blood flow within the islet is still debatable. Nevertheless, three different models have been proposed[74]. One model using *in vivo* imaging in the mouse observed that the direction of blood is going from the β-cell rich islet core towards the non β-cells in the periphery[73]. However, the question still remains as to whether blood flow in human islets follows the same route, as the distribution of α/β-cells differ between human and mouse islets. In addition, it appears that islet blood flow is regulated by a neuroendocrine loop and endothelial mediators such as nitric oxide[75].

Dysfunction of the islet vasculature and islet inflammation has been implicated in the pathogenesis of type 2 diabetes. Several mechanisms have been proposed, for instance, increased islet capillary blood pressure and perfusion have been observed in short term hyperglycemia. This is likely due to a mechanism mediated by nitric oxide[76,77]. However, a sustained increase in capillary pressure and perfusion would lead to endothelial cell injury and thickening of the capillary wall, eventually resulting in decreased islet perfusion followed by impaired insulin secretion[78,79]. Endothelial cell activation and oxidative stress have been observed to lead to recruitment of monocytes to islets, which in turn cause islet dysfunction by secreting proapoptotic cytokines[80]. Studies in a Zucker type 2 diabetic rat model also suggested a pathogenic role of altered islet vasculature in the onset of type 2 diabetes[81]. In summary, development of an abnormal islet vasculature and islet vascular defects may also contribute to the development of type 2 diabetes.

2.2 Lipid Transport and Islets of Langerhans

2.2.1 Endothelial Lipid Transport

During fasting and feeding cycles, in order to sustain energy homeostasis, larger metabolites *e.g.* glucose and LCFAs must be transported from storage sites to different organs for utilization. Blood vessels are the major channels to enable this transportation. However, the mechanisms that regulate lipid transport is still poorly understood. Endothelial cells are

constantly exposed to circulating lipids and free fatty acids (FFAs) must penetrate the endothelial barrier in order to enable uptake in underlying tissue cells. Originally, a model of a flip-flopping mechanism was proposed, in which the hydrophobic FAs could diffuse freely through the plasma membrane[82]. However, evidences from several other studies indicated the existence of an active FA trans-endothelial transport system[83]. Moreover, another study observed that high concentration of FFAs could disturb the endothelial barrier, which facilitates the movement of low density lipoprotein (LDL) into arteries[84]. Hence, the detailed molecular mechanisms that govern endothelial FA uptake is still controversial to date and collective data indicate that both protein-mediated uptake and non-specific uptake may be involved[85,86]. Several proteins involved in endothelial FA uptake have been characterized, for example the scavenger receptor CD36 and the FATP protein family (FATPs 1-6)[83].

CD36, also called fatty acid translocase (FAT), is expressed by monocytes, thrombocytes and endothelial cells in most metabolically active tissues, such as cardiac and skeletal muscle, and white and brown adipose tissue[87]. CD36 has an important function in recognizing and binding different lipids and lipid molecules[88-91], and to facilitate cellular uptake of LCFAs[92,93]. Although the precise biological function of CD36 in endothelial cells is still under debate, several studies have demonstrated its role in endothelial lipid transport[94-96]. For example, the transcription factor PPARγ upregulates *Cd36* expression[94,95]. Absence of CD36 expression and reduced FA uptake to white adipose tissue have accordingly been observed in mice with endothelial specific *Pparg* deletion, which highly resemble the phenotype of *Cd36* knockout mice[96]. In another interesting study, cold exposure could potently induce *Cd36* expression in brown adipose tissue to maintain thermogenesis by increasing cellular TAG accumulation[60]. A recent study also identified a FA binding site in CD36, partly overlapping with the binding site of oxidized LDL[97].

The FATP family consists of 6 highly conversed mammalian multi-transmembrane proteins (FATPs 1-6)[98-100]. Initially, the first FATP was discovered by murine adipocyte cDNA expression cloning as a protein that facilitated LCFA uptake[99], and subsequently additional FATP family members have been discovered and renamed[101]. FATP1 is mainly expressed in adipose tissue and heart[19,99]. FATP2 is expressed almost exclusively in the liver and kidney[99,102]. FATP3 seems specifically to be expressed within the vasculature[55,103]. FATP4 has a similar expression pattern as FATP1 but is also expressed in the intestine and endothelium. FATP4 is the only FATP that is expressed in the intestine[19,104]. Endothelial cells mainly express FATP3,

however, other FATPs are believed to be expressed at lower levels[105]. All FATPs have been demonstrated to increase cellular LCFAs uptake both *in vivo* and *in vitro*[19,106,107]. For instance, FATP3 and FATP4 co-expression in cultured endothelial cells lead to increased LCFAs uptake[19]. However, some studies argue that the effect *in vivo* is most likely due to the assistance from several other proteins which facilitates the uptake of LCFAs into endothelial cells[108].

2.2.2 Lipid Transport in Pancreatic Islets

FAs play an important role in β-cell survival and secretory function. However, excessive exposure of β-cells to FAs will result in a series of negative effects, such as impaired insulin gene expression and secretion, and initiation of β-cell apoptosis, which all contribute to the development of β-cell failure[109]. Therefore, the comprehension of how FAs are being transported into β-cells lays the foundation for identifying future drug targets for β-cell lipotoxicity.

Depending on the chain length, FAs can cross plasma membrane into β-cell either by diffusion (e.g. short chain FAs (SCFAs)) or via cell surface transporter CD36 (LCFAs)[110]. Upon entry, fatty acyl-CoA synthetase activates incoming FAs to generate acyl-CoA, which then further undergoes β-oxidation for energy production[111]. Alternatively, acyl-CoA enters the glycerolipid/free fatty acid cycle to produce triglycerides (TGs), diacylglycerol (DAG), monoacylglycerol (MAG) and other lipid components[112], or participates in the sphingolipid synthesis pathway to generate metabolites such as ceramides and sphingosine-1 phosphate[110]. In addition to the exogenous source of FAs, β-cell FAs can be derived from hydrolysis of intracellular TGs and phospholipids as lipoprotein lipase is also expressed in pancreatic islets[113,114].

The process of transmembrane FA uptake into β-cells is mediated by plasma membrane fatty acid-binding proteins ($FABP_{pm}$), which dissociates the FA from albumin at plasma membrane prior to binding to CD36[115,116]. Upon entering β-cell, cytoplasmic FABPs bind the FA and transport it to specific cellular compartments for further utilization[117]. Furthermore, this process has also been demonstrated in human islets and in an *in vitro* study using INS-1 cells[118,119].

2.2.3 Lipid Signaling in Pancreatic Islets

In addition to the abovementioned transmembrane transport machinery, a group of G-protein coupled receptors on the β-cell surface, also referred to as FFA receptors (FFARs), have recently been discovered to function as signaling receptors for FAs, affecting a number of intracellular lipid signaling pathways. Currently four FFA receptors, FFAR1 (GPR40), FFAR2 (GPR43), FFAR3 (GPR41) and FFAR4 (GPR120) have been shown to be expressed in rodent and human β-cells[120]. Interestingly, the FFARs (1-4) exhibit chain length specificity (Figure 3). For instance, FFAR1 and FFAR4 specifically bind to medium and long-chain FAs, whereas FFAR2 and 3 only bind to short-chain FAs[120].

Upon binding of the FA to its respective receptor, the complex exerts different effects on β-cell and systemic glucose homeostasis. For instance, binding of eicosatrienoic acid (C20:3) to FFAR1 (GPR40) facilitates glucose stimulated insulin secretion in β-cells[121,122]. However, opposing effects were observed in a number of *in vivo, in vitro* and human studies regarding the downstream effects of FFAR1. An *in vitro* study demonstrated protective effect of FFAR1 against lipotoxicity in rat insulinoma cells (INS-1)[123], and overexpression of FFAR1 in β-cells improved glucose tolerance and glucose-stimulated insulin secretion (GSIS) *in vivo*[124]. In line with these observations, a single nucleotide polymorphism in the *FFAR1* locus in humans is associated with insulin secretory dysfunction[125]. Conflicting with these results, a study by Steneberg et al. showed that loss of FFAR1 in mice protected against obesity-induced hyperglycemia, glucose intolerance and hyperinsulinemia, whereas FFAR1 overexpression in β-cells resulted in β-cell dysfunction and diabetes[126]. Yet in another study, chronic exposure of palmitate to human islets resulted in decreased insulin content and secretion, and these effects could be prevented by a FFAR1 antagonist, ANT203[127]. The discrepancies in the different studies might be attributed to the difference in study design, such as duration of exposure, and species differences.

FFAR4 (GPR120) is the receptor for unsaturated FFAs and LCFAs and shows high binding affinity to docosahexaenoic acid (DHA) and α-linoleic acid, which are both ω-3 FAs[128]. Activation of FFAR4 increases glucagon-like peptide-1 (GLP-1) secretion from intestinal enteroendocrine L cells which in turn enhances insulin release from β-cells[128] and protects β-cell from apoptosis via activation of phosphoinositide 3-kinase-Akt (PI3K) and extracellular signal regulated kinase (ERK)[129]. FFAR4 knockout mice are furthermore glucose intolerant and an inactivating mutation in *FFAR4* has been linked with obesity in human[130].

FFAR2 and 3 (GPR43, 41) are receptors for SCFAs, particularly FAs consisting of 3, 4 and 5 carbons. Activation of these receptors by SCFAs has been shown to inhibit insulin secretion via G protein coupling[131] and loss of both receptors in β-cells improve glucose tolerance and insulin release in high fat diet mice[131]. However, FFAR2 and 3 are essential for SCFA mediated GLP-1 secretion from intestinal enteroendocrine L cells, and consequently the global knockout of both receptors resulted in glucose intolerance *in vivo*[132]. Thus, the therapeutic strategy by targeting these receptors is debatable.

A number of nuclear receptors have also been demonstrated to function as receptors for FFAs. PPARα can function as a receptor for LCFAs and upon activation, PPARα increases FA oxidation and hence reduces lipid storage, protecting from lipotoxicity[133]. Additionally, farnesoid X receptor (FXR), a bile acid receptor, is also shown to regulate β-cell function in human islets[134].

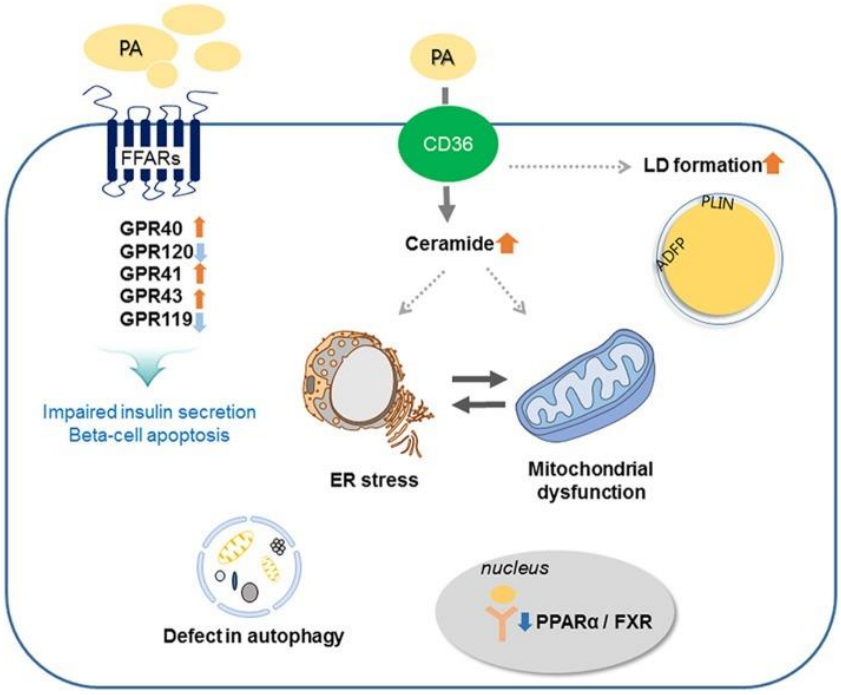

Figure 3. Schematic diagram of fatty acid uptake into pancreatic β-cells and potential consequence

Palmitate (PA) transmembrane transport by CD36 results in formation of increased ceramide and formation of lipid droplets (LD), which trigger cell stress responses, such as ER stress, mitochondrial dysfunction and defective autophagy. PA also activates FFA receptors resulting in impaired insulin secretion. *(Modified image from Oh et. al, Frontiers in Endocrinology, 2018[3])*

2.3 Lipotoxicity in Pancreatic Islets

Obesity is one of the causes for the development of type 2 diabetes and commonly associated with insulin resistance[135]. In physiological condition, β-cells are very sensitive to rising circulating blood glucose and respond by releasing insulin to maintain a normal blood glucose level. Several nutrients can also trigger insulin release such as amino acids and fatty acids.

Depending on the concentration and duration, FAs can exert both positive and negative effects on β-cells, such as β-cell survival, insulin secretion and membrane composition[136]. Increased amounts of circulating FAs as a direct result of increased lipid accumulation and lipolysis in peripheral tissue is commonly associated with obesity[137]. Moreover, chronic exposure of FAs to islets severely suppresses the synthesis of proinsulin, impairs glucose stimulated insulin secretion from islets and also decreases insulin storage, leading to apoptosis and eventually results in β-cell failure[136].

Dysfunction of the secretory capacity of β-cells and insufficient insulin production due to detrimental loss of β-cell mass eventually leads to the development of type 2 diabetes. Pancreatic islet lipotoxicity is considered to be one of the main causes for the onset and progression of this pathological process. Several likely mechanisms of lipotoxicity-induced β-cell dysfunction have been proposed over the years; in this chapter, endoplasmic reticulum (ER) stress, mitochondrial dysfunction and defective autophagy are mainly discussed.

2.3.1 ER Stress

In normal conditions, pancreatic β-cells depend on a highly active ER to satisfy the high secretory demands of insulin, as more than half of the total protein is synthesized in the ER[138]. Therefore, β-cell function is particularly vulnerable to ER stress, which subsequently would trigger unfolded protein response (UPR) on ER.

FA-induced ER stress is mainly mediated through interference with protein processing and trafficking[139] or dysregulation of Ca^{2+} efflux[140]. Disturbance of Ca^{2+} regulation in the ER results in incorrect protein folding, subsequently causing ER stress. Several studies have shown that long-term exposure of saturated FAs could inhibit ER vesicle trafficking in β-cells, confirming the theory that impaired protein trafficking could result in ER stress[141]. Palmitate (C16:0), the most common saturated FA, was recently shown to induce ER stress via depletion of ER Ca^{2+}[142]. And in the case of prolonged ER stress due to β-cell lipotoxicity,

failure of repairing incorrectly folded proteins may lead to β-cell apoptosis[143]. Hence, current findings indicate that lipotoxicity plays a unique role in ER stress mediated β-cell failure in the development of type 2 diabetes.

2.3.2 Mitochondrial Dysfunction

Dysfunction of mitochondria have been strongly correlated with impaired β-cell function. Under physiologically low glucose conditions, β-cells utilize FAs to generate ATP via β-oxidation to maintain their energy demands[144]. In obesity, increased availability of circulating FAs augment β-oxidation and oxidative phosphorylation in mitochondria, which result in excessive reactive oxygen species (ROS) production[145]. Pancreatic β-cells have a limited capacity for handling oxidative stress, therefore mitochondria, where the majority of ROS are produced, carries a higher risk for suffering from oxidative injury[146]. In normal conditions, only 0.1% of consumed oxygen generates ROS due to inefficient electron transport[147]. However, in diabetes and other pathological conditions, exacerbated ROS formation eventually results in oxidative stress and damaged mitochondria leading to β-cell failure[146]. Studies have shown that long term exposure of rat islets to saturated FAs induces β-cell apoptosis and this effect could be rescued by addition of antioxidants, suggesting that the toxic effect of FAs on β-cells could be mediated by oxidative stress[148].

2.3.3 Defective Autophagy

Autophagy is an important cellular mechanism that allows the safe removal of dysfunctional cellular components, protein aggregates and pathogens. Recent research on the connection between intracellular lipids and autophagy in pancreatic islets indicated that autophagy also plays a role in the pathogenesis of type 2 diabetes. Under normal physiological conditions, autophagy is important for the maintenance of cellular quality control in β-cells, and this activity is indispensable for proper β-cell architecture and function[149]. Mice with deficient β-cell autophagy display with impaired glucose tolerance and insulin secretion, decreased β-cell proliferation, increased apoptosis and accumulation of protein aggregates[150]. The mice also exhibit accumulation of autophagosomes when evaluated in diabetic settings[150]. An *ex-vivo* study using non-diabetic human islets demonstrated build-up of autophagic vacuoles and increased apoptosis after exposure to saturated FAs[151]. In addition, several *in vitro* studies of INS-1E cells also observed increased amounts of autophagosomes when treated with saturated FAs, and this effect was also accompanied by impaired lysosome formation[152].

Electron microscopic analysis of human islets from type 2 diabetic patients also revealed excessive autophagosomes which might be caused by impaired lysosome formation[151]. These data indicate that impaired β-cell autophagy may be more susceptible to lipotoxicity. However, further studies are needed to elucidate the specific role of autophagy in lipotoxicity induced β-cell failure.

III

ISLET INTRAVITAL IMAGING

3.1 Development and History

The pathophysiology of type 2 diabetes is owing to the development of β-cell dysfunction, reduction in β-cell mass and insulin resistance[153-155]. Major efforts have been investing into understanding the mechanisms of underlying causes of these processes within the research field. Notably, functional differences of pancreatic islets *in vitro* versus *in vivo* have been documented[156,157]. However, one major challenge is the lack of appropriate noninvasive animal models to study dynamic changes in islet mass and function during disease progression of i*n vivo*. Approaches, such as *in vivo* optical monitoring of β-cell mass using MRI[158] lacks the spatial resolution required to study physiology at cellular level. Therefore, development and application of suitable and accessible model systems to enable *in situ* visualization and assessment of islet function *in vivo* is warranted.

The anterior chamber of the eye (ACE) provides an optically accessible site with a suitable innate environment in terms of oxygen supply, nerve innervation and immune system[159]. In 2008, Speier et al. developed the technique combining transplantation of pancreatic islets into the ACE and confocal/multiphoton microscopy to visualize and study islet function/behavior *in vivo*[160]. This minimal-invasive method makes it possible to visualize pancreatic islets under

different conditions in real-time[160]. Since its development, the islet intravital imaging setup has been widely used to address various questions within diabetes research.

3.2 Islet Intravital Imaging Technique and Application

The islet intravital imaging technique consists of two parts: First, freshly isolated or cultured pancreatic islets are transplanted into the ACE. This is followed by imaging of the engrafted islets at various timepoints post transplantation[161]. Depending on the research objectives, islets from donor mice harboring specific genetic modifications allowing for example direct visualization of a particular cell type (*e.g.* vessel specific labeling) can be transplanted to recipient mice[159]. To enable subsequent imaging of engrafted islets in the ACE, the anesthetized recipient mouse needs to be fixated on an imaging platform using a head holder, and then the eye is stabilized with an eyeball holder and oriented towards the microscope lens (Figure 4)[161].

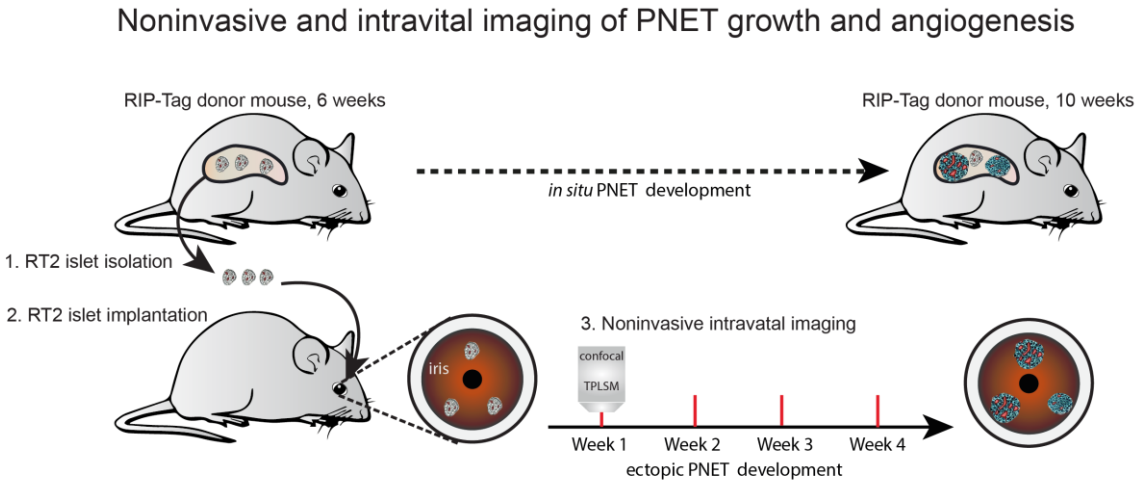

Figure 4. Graphic illustration of noninvasive intravital imaging technique

1. Isolation of tumorigenic RT2 islets. 2. Transplantation of isolated RT2 islets into the anterior chamber of the eye. 3. Weekly multiphoton/confocal intravital imaging of islet tumor progression. *(Modified image from Balan et. al, Scientific Reports, 2019[2])*

The islet intravital imaging technique provides several benefits. Firstly, the minimal-invasive nature of this technique by using the eye as a natural window and therefore removing the anatomical barrier to enable *in vivo* imaging. Secondly, the high spatial imaging resolution that can be obtained by confocal and multiphoton imaging. Thirdly, the preservation of

morphological and functional characteristics of engrafted islets, which increase the relevance of the experimental observations both in physiological and pathological conditions[157,159,160].

Since the development of this technique, substantial amount of knowledge has been accumulating regarding islet engraftment. Various studies have demonstrated that implanted islets rapidly integrate in the ACE environment and acquire adequate vascularization and innervation[162-165]. Furthermore, this technique is not limited only to islet transplantation, a recent study has also successfully demonstrated transplantation of pancreatic buds from 10.5-day mouse embryos for the purpose of studying pancreatic islet development[166]. Furthermore, a number of studies have been using this technique to investigate the *in vivo* dynamic profile of β-cell Ca^{2+} signaling, function and insulin resistance during the development of type 2 diabetes[167-169].

In study III of this thesis, we subjected islets from a mouse model of pancreatic neuroendocrine tumor (Rip1Tag2, RT2 for short) to intravital imaging to study the development of tumor vasculature growth and dynamics with VEGF-B genetic manipulations. The RT2 mouse model is a well characterized pancreatic tumor model, in which simian virus 40 large T-antigen (Tag) oncogene is expressed under the rat insulin promoter. From birth, a multifocal stepwise β-cell carcinoma will develop in pancreatic islets[170,171]. Detailed description is described in paper III.

IV

ENDOTHELIAL Wnt/β-Catenin SIGNALING

4.1 Introduction to Wnt/β-Catenin Signaling

The term Wnt originated from the combination of Wingless and Int-1[172]. The Wnt signaling cascade is conducted either in paracrine or autocrine fashion and is highly conserved between species[173,174]. Currently three Wnt pathways have been identified: The canonical Wnt/β-catenin pathway, the noncanonical planar cell polarity pathway and the noncanonical Wnt/Ca^{2+} pathway. The canonical Wnt/β-catenin pathway is the most studied and regulates gene transcription[175], the noncanonical planar cell polarity pathway regulates cytoskeletal organization and coordinate the polarization of the cell[176], and the Wnt/Ca^{2+} regulates cellular calcium signaling and affects various intracellular signaling[177,178]. Wnt signaling pathways play a vital role in embryonic development, and is involved in a number of cellular processes such as cell fate specification, cell proliferation and migration, and genetic mutations affecting Wnt signaling have been implicated in cancer development[175,179]. In this chapter, only the canonical Wnt/β-catenin pathway will be discussed.

Activation of Wnt/β-catenin signaling requires the binding between Wnt and its receptor Frizzled (FZD) which leads to translocation of β-catenin to the nucleus and subsequent transcription of Wnt target genes[174]. In the absence of a Wnt ligand, β-catenin is targeted for proteolytic degradation, regulated by a destruction complex consisting of the tumor suppressor protein Axin, which functions as a scaffold for the complex, the tumor suppressor

protein APC, and two kinases, the casein kinase 1(CK1) and glycogen synthase kinase 3 (GSK3)[180]. CK1 and GSK3 sequentially phosphorylate β-catenin, and phosphorylated β-catenin is then recognized by the E3 ubiquitin ligase complex β-TrCP, which it ubiquitinates β-catenin and targets it subsequently targeted by proteasomal degradation, which resulting in rapid destruction of β-catenin[181].

In the activated state, Wnt binds to its receptor FZD, and FZD then recruits the cytosolic effector protein Dishevelled (Dvl), which enables the binding of Axin to the plasma membrane. Axin together with other bound kinases phosphorylate low density lipoprotein receptor-related protein 5/6 (LRP5/6) in the plasma membrane, which provides further binding sites for Axin to form a stable Wnt-receptor-Dvl-Axin complex[182]. As a result, the destruction complex becomes inactivated, leading to accumulation of β-catenin in the cytosol and translocation to the nucleus where it interacts with DNA and T cell factor (TCF), resulting in transcription of Wnt target genes[182,183].

Although the general theory of inner working of β-catenin destruction complex is widely accepted by the scientific community. New knowledge/evidence are emerging and continue to uncover additional intricate connections and regulations between each player. Along with the development of novel technologies, such as CRISPR-Cas genome editing system, it is likely to reveal more answers to many of the unsolved questions within the Wnt field in the foreseeable future.

AIMS OF THE THESIS

The overall aim of this thesis is to investigate the role of VEGF-B in pancreatic β-cell physiology in the context of β-cell function, glucose homeostasis, islet lipid handling and angiogenesis. The specific aims are:

1. To investigate the effect of β-cell VEGF-B deficiency on pancreatic islet physiology, focusing on β-cell function and islet vascularization, and potential consequences on systemic glucose homeostasis and islet lipid content.

2. To study the impact of transgenic overexpression of the human VEGF-B_{167} isoform in pancreatic islet β-cells on islet physiology and vascularization, and potential effects on systemic glucose and lipid homeostasis.

3. To explore the effect of human VEGF-B_{167} overexpression on pancreatic islet tumor progression and tumor angiogenesis *in situ* using an in-house developed intravital high-resolution imaging platform.

4. To elucidate the role of endothelial Wnt/β-catenin signaling in central nervous system angiogenesis and potential crosstalk with VEGF signaling pathways.

RESULTS AND DISCUSSION

Study I. VEGF-B ablation in pancreatic β-cells upregulates insulin expression without affecting glucose homeostasis or islet lipid uptake

Increased lipid uptake and handling can result in β-cell lipotoxicity, which contributes to β-cell dysfunction and development of type 2 diabetes mellitus. VEGF-B signaling in endothelial cells have been shown to increase endothelial fatty acid transcytosis via paracrine regulation of FATP-3 and -4, contributing to lipotoxicity in peripheral tissues such as heart and skeletal muscle. Systemic inhibition of VEGF-B results in improved metabolic performance in diabetic mouse models, correlating with improved insulin sensitivity and glucose tolerance[55]. However, the specific role of VEGF-B in islet β-cell physiology has yet to be examined. In this study, we generated and characterized a novel mouse model where VEGF-B in pancreatic β-cells has been selectively deleted using the Cre/LoxP system to investigate the tissue specific role of VEGF-B. We assessed whether ablation of VEGF-B in pancreatic β-cells would affect islet function, systemic glucose homeostasis and more importantly whether deficiency of VEGF-B would affect pancreatic islet lipid uptake, of relevance for β-cell lipotoxicity.

VEGF-B is ubiquitously expressed in pancreas

To address these questions, we started out by generating a mouse strain where *Vegfb* is selectively deleted in pancreatic β-cells. To achieve this, we crossed *Vegfb* floxed mice ($Vegfb^{fl/fl}$) with RIP-$Cre^{+/-}$ mice, to generate $Vegfb^{fl/fl}$/RIP-$Cre^{+/-}$ mice. To validate β-cell specific *Vegfb* deletion, RNA was isolated from $Vegfb^{fl/fl}$/RIP-$Cre^{+/-}$ islets, and real-time quantitative PCR indicated 80% reduction of total *Vegfb* transcripts. Additionally, we also applied RNA *in situ* hybridization (RNAscope) to visualize the distribution of *Vegfb* transcripts. We observed that *Vegfb* is abundantly expressed in both the endocrine and exocrine parts of the pancreas in control mice, and that a visible reduction of *Vegfb* transcripts was confined to the islets in β-cell *Vegfb* deficient mice. Together these data indicated that *Vegfb* is abundantly expressed in pancreas and that the efficient reduction

of islet *Vegfb* transcripts in *Vegfb*$^{fl/fl}$/RIP-Cre$^{+/-}$ mice points to that β-cells are the major source of islet VEGF-B.

Deficiency of *Vegfb* in pancreatic β-cells results in increased insulin gene expression

To evaluate the effect of *Vegfb* deficiency on islet gene expression, we performed real-time PCR on islet RNA retrieved from *Vegfb*$^{fl/fl}$/RIP-Cre$^{+/-}$ and control mice. Interestingly, insulin gene *Ins2*, but not *Ins1*, was upregulated upon ablation of *Vegfb* in β-cells and conversely, we observed a trend of downregulation of islet glucagon gene expression (*Gcg)* when compared to the RIP-Cre control group but failed to reach significance when compared to the *Vegfb*$^{fl/fl}$ control. Furthermore, we also evaluated islet gene expression after 20-weeks of HFD feeding. However, islet insulin and glucagon expression levels remained similar across the groups.

Unlike other species, rodent insulin is encoded by two-gene system, *Ins1* and *Ins2* respectively[184,185]. *Ins1* is specifically to rodent, whereas *Ins2* has greater similarities to the *Ins* gene in other mammalian species. Insulin gene expression is tightly controlled by blood glucose via transcriptional regulation[186], a number of transcriptional factors such as Pdx-1, PAX6, NueoD1 and MafA have been shown to regulate insulin expression in β-cell[187-189]. Our finding of *Vegfb* deficiency in β-cells only upregulates islet *Ins2* expression might suggest a potential link between *Vegfb* and transcriptional factors of *Ins2*. As PGC-1α regulates VEGF-B expression[32], and PGC-1α has also been implicated in re-programming of Pdx-1 in adult β-cell[190], hence it is plausible to speculate the possibility. Further studies are needed to elucidate aforementioned regulation machinery.

β-cell specific *Vegfb* deficiency does not affect islet function or glucose homeostasis under chow and HFD diet condition

To evaluate the effect of β-cell *Vegfb* deficiency on islet function, we examined glucose stimulated insulin secretion (GSIS) on isolated islets from all experimental groups. We found that β-cell *Vegfb* deficiency causes increased basal insulin release under low glucose conditions. However, this effect was diminished when stimulated with high glucose. Additionally, deficiency of *Vegfb* in β-cells did not alter total islet insulin content, or the pool of readily released insulin granules. When challenged by HFD, we did not observe any difference in GSIS, islet insulin content and KCl stimulated insulin secretion.

To investigate the effect of β-cell *Vegfb* deficiency on systemic glucose homeostasis, we measured a range of glucose related parameters, such as postprandial/fasting blood glucose, postprandial plasma insulin, glycated hemoglobin A_1C, as well as performed glucose and insulin tolerance tests. Although we found significant changes in mice with β-cell *Vegfb* deficiency in postprandial/fasting blood glucose, postprandial plasma insulin level and insulin tolerance test when compared with RIP-Cre control groups, however, those parameters failed to reach statistical significance when compared to $Vegfb^{fl/fl}$ control mice. Similar set of tests were also performed on HFD mice, and consistent with previous observations, mice on HFD developed significant weight gain and mildly increased blood glucose, compared to age-matched mice on chow diet. However, all tested parameters related to glucose homeostasis showed insignificant difference in mice with β-cell *Vegfb* deficiency when compared to the control groups. Therefore, we concluded that deficiency of *Vegfb* in pancreatic β-cells does not affect islet function or overall glucose homeostasis under chow and HFD conditions, and increased insulin release under low glucose condition are likely attributed by cell autonomous effect and potentially conferred by upregulated islet *Ins2* gene expression.

Pancreatic islet VEGF-B expression is not affected by increased metabolic activity and β-cell *Vegfb* deficiency does not affect lipid uptake in islets

To understand the role of VEGF-B deficiency for islet lipid uptake and also the effect of β-cell *Vegfb* deficiency on downstream signaling of VEGF-B, we examined mice under both chow and HFD feeding conditions. Real-time PCR revealed that islet *Vegfb* transcripts were not increased after HFD treatment, suggesting that *Vegfb* expression is not regulated by increased metabolic activity in pancreatic islets. Plasma TG levels were similar between $Vegfb^{fl/fl}$/RIP-$Cre^{+/-}$ mice and the controls groups both under chow and HFD. When comparing the TG level in mice fed on chow *vs.* HFD, increased plasma TG were found in all four genotypes. However, only RIP-$Cre^{+/-}$ and $Vegfb^{fl/fl}$/RIP-$Cre^{+/-}$ groups reached a significant increase in circulating TG levels in HFD as compared to chow condition. To further examine the effect of β-cell *Vegfb* deficiency on islet lipid uptake, we measured islet ceramide content and *Plin2* gene expression, as both readouts represents possible fates of FA transportation into β-cells. Islet ceramide levels and *Plin2* gene expression were not affected by β-cell *Vegfb* deficiency under chow or HFD conditions. Additionally, *Cd36* and VEGF-B signaling downstream target genes encoding FATP3 and 4 were also unaffected.

Collectively, in this study we demonstrated that although *Vegfb* deficiency in islet β-cells correlated with upregulation of insulin gene expression, the effect on islet function, systemic glucose homeostasis or islet lipid uptake was negligible suggesting local paracrine VEGF-B signaling in pancreatic islets has limited therapeutic potential on overall glucose homeostasis and also as a target for reducing islet lipotoxicity.

Study II. Overexpression of VEGF-B in pancreatic β-cells does not affect glucose homeostasis but increases plasma triglycerides

Previous investigations have demonstrated a limited role for VEGF-B in angiogenesis[2,21,53,54,56,191], but instead has an essential role for endothelial fatty acid transcytosis and lipid accumulation in tissues with high metabolic capacity been described[55]. However, the tissue specific role of VEGF-B signaling and whether local VEGF-B signaling would affect target organ function or systemic metabolic homeostasis remains unsettled. In this study, we have utilized a previously established mouse model where the human VEGF-B_{167} isoform is transgenically overexpressed in β-cells, and we have assessed the effect under both chow and HFD conditions.

Transgenic overexpression of VEGF-B does not affect islet function or morphology

We first validated the transgenic VEGF-B expression in pancreatic islets using real-time PCR. In comparison to wildtype control mice, islets from β-cell VEGF-B overexpressing (RIP1-VEGFB) mice showed similar amounts of mouse *Vegfb* transcripts, whereas islets from VEGF-B global knockout (*Vegfb*$^{-/-}$) mice, as expected, had nearly no detectable mouse *Vegfb* expression. Furthermore, we observed that human *VEGFB* was about 40-fold higher expressed than mouse *Vegfb* in islets from RIP1-VEGFB mice, indicating an efficient transgenic overexpression of human *VEGFB*.

Next, we evaluated the islet function and morphology in wt and RIP1-VEGFB mice. Under both chow and HFD condition, GSIS revealed no difference in response to low and high glucose stimulation between wt and RIP1-VEGFB islets, and KCl stimulated insulin secretion also showed no difference. Taken together, these data suggested that β-cell VEGF-B overexpression does not affect islet secretory function. To further evaluate whether overexpression of VEGF-B affects pancreatic islet morphology, tissue sections from cryopreserved pancreata were subjected to immunofluorescence stainings with

insulin, glucagon and CD31 antibodies. Interestingly, we observed an increased vessel area (CD31 staining) in RIP1-VEGFB mice, which was in line with the observations from previous studies [2,21]. This effect could be explained by the VEGF-B/VEGFR1 decoy theory proposed by Robcius et. al[53].

Systemic glucose homeostasis is not affected by VEGF-B overexpression in pancreatic β-cells

To address the question whether VEGF-B overexpression in pancreatic β-cell would affect systemic glucose homeostasis we measured a series of glucose parameters under both chow and HFD diet condition. We found that body weight, postprandial/fasting blood glucose, postprandial plasma insulin and glucose and insulin tolerance tests were all similar between wt and RIP1-VEGFB mice, suggesting that pancreatic β-cell overexpression of VEGF-B does not affect systemic glucose homeostasis.

Transgenic overexpression of VEGF-B in pancreatic β-cells increases plasma triglycerides

We unexpectedly found a significantly increased plasma TG level in RIP1-VEGFB mice. This phenotype was evident in both chow and HFD feeding condition. Interestingly, the increased TG level only existed in male mice, as plasma TG in female RIP1-VEGFB mice were of similar level to their wt control (data not shown). Currently, the particular mechanism of transgenic overexpression of VEGF-B in β-cells causing increased plasma TG is unknown. However, there are several possible angles that might help to explain this phenotype. As plasma TG is represented by both chylomicron and very low-density lipoprotein (VLDL), it is reasonable to speculate that pancreatic overexpression of VEGF-B can influence the formation of chylomicrons or VLDL, or alternatively, affecting activation of lipolysis. Further studies are warranted to elucidate this effect.

In summary, in this study we found that transgenic overexpression of VEGF-B in β-cells does not affect islet function and systemic glucose homeostasis. However, locally expressed VEGF-B in pancreatic β-cells causes increased vessel area and increased plasma TG level, a phenotype warranting further studies.

Study III. Noninvasive intravital high-resolution imaging of pancreatic neuroendocrine tumours

In study III, we explored the role of the VEGF-B in tumor angiogenesis by employing a novel intravital 3D imaging platform developed in-house on islets from the pancreatic neuroendocrine tumor model Rip1Tag2, hereafter denoted RT2. The core principle of this technique is to transplant islets from mice with different genetic background (normal, fluorescent, tumorigenic) into the anterior chamber of the eye (ACE) of a recipient mouse, and using the eye as a natural window to image the engrafted islets with focus on vascularization, overall growth of tumor islets, the effect of VEGF-B on tumor angiogenesis and testing pharmacological inhibition with a topical approach and its effects on tumor angiogenesis.

Pancreatic neuroendocrine tumor characterization

We first characterized RT2 tumor islet growth with ACE intravital imaging. Compared to normal islets, RT2 tumor islets grew rapidly, and were characterized by a tortuous vasculature. In addition, we observed that capillaries in RT2 tumor islets have irregular shapes, wider diameter and irregular blood flow. To further monitor tumor angiogenesis during cancer progression, we cross-bred RT2 mice with a number of different fluorescent reporter mice (EYFP, tdTomato) and injected fluorescence dyes to better visualize tumor angiogenesis and tumor cell behavior.

We further developed a tool for 3D quantification of ACE engrafted tumor progression, capable of calculating the tumor volume by 3D surface projection rendering, as well as by digital skeletonization of the islet vasculature assessment and quantify the number of branch/end points, segment diameter, length and number.

Sunitinib treatment resulted in tumor regression and reduced tumor growth

Next, we validated the feasibility of pharmacological treatment of RT2 tumor in our ACE platform, with the objective of evaluating the effect of drug treatment of RT2 tumors in real-time. Two weeks after transplantation, pre-oncogenic RT2 islets were subjected to Sunitinib treatment for another two weeks and we observed strong tumor vessel regression in response to Sunitinib. Further quantification of Sunitinib treated RT2 tumor islets showed significantly decreased vascular volume, length and branch points. Control treated RT2 tumor islets continued to grow till the end of study, whereas Sunitinib treatment halted RT2 tumor growth. Taken together, these observations demonstrated the potential of the ACE platform for revealing novel information during the course of cancer

treatment, which could be beneficial for developing new strategies for future cancer treatment.

Overexpression of VEGF-B reduces tumor growth but increases tumor angiogenesis

Based on previous studies, the role of VEGF-B in angiogenesis seem to be restricted to pathological conditions[26]. An earlier study found that overexpression of VEGF-B in RT2 tumor islets resulted in reduced tumor volume[21]. Endpoint histological analysis of VEGF-B overexpressing RT2 islets revealed furthermore no major vascular effects besides increased capillary diameter[21]. To gain further understanding of the role of VEGF-B in tumor angiogenesis, we crossed RIP1-VEGFB mice (mice from Paper II) with RT2 mice to generate double transgenic RT2-VEGFB mice.

Four weeks after ACE transplantation, we observed a reduction in tumor expansion in VEGF-B overexpressing RT2 islets (RT2-VEGFB). The tumors exhibited on the other hand an augmented angiogenic response. Further 3D quantification analysis indicated that RT2-VEGFB tumors display with increased vascular volume, length and number of branching points. However, vessel diameter was similar in RT2-VEGFB islets when compared to control RT2 islets. Interestingly, 3D quantification analysis of non-tumorigenic RIP1-VEGFB islets implanted into the ACE also showed increased vascular volume, number of branching points, as well as wider diameter, compared to control islets.

In summary, in this study we further developed the ACE platform and complemented with a more detailed quantification protocol, demonstrating the feasible application of the ACE platform in evaluating the efficacy of pharmacological treatments on tumor islets. More importantly, we showed that overexpression of VEGF-B suppresses pancreatic tumor islet growth and that this was accompanied by increased tumor angiogenesis.

Study IV. Endothelial β-catenin signaling supports postnatal brain and retinal angiogenesis by promoting sprouting, tip cell formation, and VEGFR2 expression

In this study, we explored the contribution of endothelial Wnt/β-catenin signaling in postnatal central nervous system (CNS) angiogenesis. Activation of endothelial β-catenin signaling has an essential role in vascularization of brain and retina and mutations in the

endothelial β-catenin pathway has been linked to impaired vascularization, blood-brain barrier dysfunction and a number of CNS diseases[192].

To visualize active β-catenin signaling in postnatal cerebral vasculature, we utilized a reporter mouse line where β-catenin transcriptional activity induces GFP expression and we observed that endothelial β-catenin signaling was very active throughout postnatal cerebral development. To better understand the role of Wnt/β-catenin in CNS angiogenesis, we used inducible genetically modified mice modeling activated (gain-of-function) or inactivated (loss-of-function) endothelial β-catenin signaling. Endothelial β-catenin loss-of-function in the postnatal stage (day 6) resulted in retinal hypovascularization with deficient endothelial cell proliferation and sprouting, in agreement with previous observations[193,194]. Conversely, using inducible β-catenin gain-of-function mice, we further demonstrated that endothelial β-catenin signaling regulates tip cell selection during sprouting angiogenesis in the retina.

Additionally, endothelial β-catenin signaling also actively promote blood-brain barrier formation and sprouting angiogenesis, as judged by the reduced expression of markers for these processes in β-catenin loss-of-function mice. Some of the effects may be linked to defective VEGF-A signaling as the expression of both VEGFR2 and -3 was diminished in the CNS microvasculature. Interestingly, we also found that impaired endothelial β-catenin signaling prevented pathological hypersprouting when Notch signaling was inhibited, presumably related to failure of upregulating VEGF-A/VEGFR2 signaling.

In summary, in this study we demonstrated that endothelial β-catenin is crucial for angiogenesis under low Notch signaling. We further propose that impaired endothelial β-catenin signaling result in reduced retinal and cerebral angiogenesis due to reduced VEGFR2 expression in the CNS microvasculature, demonstrating a crosstalk between β-catenin and the VEGF/VEGFR2 signaling pathway.

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